

UVB Radiation Illuminates the Role of TLR3 in the Epidermis

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UV radiation poses a significant risk to human health. The mechanisms that help repair UV-damaged cells have recently been more clearly defined with the observation that Toll-like receptor 3 can sense self RNA released from necrotic keratinocytes following UV damage. TLR3 activation in the skin induces inflammation and increases the expression of genes involved in skin barrier repair. Activation of TLR2 in the skin by commensal microbial products prevents excessive inflammation by blocking downstream TLR3 signaling. This review highlights how UV damage-induced inflammation in the skin is propagated by host products and regulated by host inhabitants.

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INTRODUCTION

Excessive exposure to UV radiation is dangerous and has significant negative effects on human health. In the year 2000, excessive exposure to UV light led to 60,000 deaths worldwide with 1.5 million disability-adjusted life years also lost (Lucas *et al.*, 2006). A majority of the reported morbidity and mortalities were linked to skin cancer; including melanoma, basal cell carcinoma, and squamous cell carcinoma, although sunburn also had a significant contribution to the 1.5 million disability-adjusted life years lost. Furthermore, the economic and psychological impact of solar aging remains unquantified.

Despite its great impact on human health, relatively little research has been dedicated toward understanding the biological events associated with this common process. Much more work has been applied to understanding the benefits of solar exposure. UV light is needed to synthesize Vitamin D, which is necessary for human health (Institute of Medicine, 2011; Ross *et al.*, 2011). Complete sun avoidance would result in diseases related to Vitamin D deficiency and it is predicted that these diseases would lead to 3,304 million disability-adjusted life years being lost (Lucas *et al.*, 2006). In addition to

generation of Vitamin D, low levels of UV radiation also enhance the permeability barrier function of the skin. Skin exposed to either UVA or UVB radiation before chemical irritant application is more resistant to damage as measured by transepidermal water loss (alkali resistance test, DMSO test, and sodium lauryl sulfate test; Lehmann *et al.*, 1991). Barrier repair is also accelerated in skin that has been previously exposed to suberythral broadband UVB radiation before tape-stripping barrier disruption. In these studies, it was also observed that antimicrobial peptide production is increased following low-level broadband UVB exposure (Hong *et al.*, 2008). Furthermore, UVB radiation can provide therapeutic benefits to patients with certain dermatological conditions. Patients with psoriasis are commonly treated with narrowband UVB (Picot *et al.*, 1992) and atopic dermatitis patients with either narrowband, broadband, or combination UVA/UVB (Jekler and Larkö 1988; Grundmann and Beissert, 2012) exposure as the beneficial effects typically outweigh the apparent long-term negative effects. UVB damage to the skin results in inflammation characterized by increased NF- κ B activation, increased inflammatory cytokines including tumor necrosis factor (TNF) and IL-6, increases in cis-urocanic acid, prostaglandins, reactive oxygen species, and DNA damage (Ichihashi *et al.*, 2003; Black *et al.*, 2008). As is the case for the negative effects of UV on the skin, the mechanisms behind the beneficial effects of UVB are still unclear. However, new insights into the functions of the innate immune system have opened a window of opportunity to better understand these important interactions.

INNATE IMMUNE RECEPTORS RECOGNIZE PRODUCTS OF CELL DEATH

The primary function of the innate immune system has historically been described as its ability to detect pathogens and to rid them from the body. These microbes have unique physical characteristics termed microbe-associated molecular patterns that allow them to be recognized as foreign by the host's array of pattern-recognition receptors (PRRs) that are present on various immune cells as well as epithelial cells including keratinocytes (Lebre *et al.*, 2007). At the most fundamental level, it is appropriate to recognize that the activation of these PRRs causes an immune response that induces inflammation and eliminates the microbe from the host. However, these same innate immune receptors have the capability of recognizing many different chemical structures in addition to those found on microbes. An important class of such non-microbial compounds is made up of certain endogenous molecules made by the host but normally separated

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Abbreviations: dsRNA, double-stranded RNA; PRRs, pattern-recognition receptors; TNF, tumor necrosis factor

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from PRRs by nature of their compartmentalization. During times of cell death, when compartmentalization of intracellular environments is disturbed, many of these host components are released into an extracellular environment, which can illicit an immune response. These endogenous molecules that can activate an immune response in a sterile environment, absent of infection, are termed damage-associated molecular patterns and, similar to microbe-associated molecular patterns, they activate PRRs resulting in inflammation and recruitment of leukocytes (Kono and Rock, 2008).

Regulated cell death in the skin is an ongoing process as keratinocytes terminally differentiate and undergo the process of cornification in order to produce the stratum corneum (Candi *et al.*, 2005). UV damage, however, can cause unintended damage to keratinocytes and subsequent cell death in the skin, often observable in histological sections as “sunburn cells”, which are accepted to be keratinocytes undergoing apoptosis (Young, 1987). High doses of UV radiation cause the formation of cyclobutane pyrimidine dimers and (6-4) photoproducts in DNA, which leads to mutations that can eventually result in skin cancer (Ichihashi *et al.*, 2003) when nucleotide excision repair fails (Rass and Reichrath, 2008). For this reason, it is essential for the health of an individual to dispose of these mutated cells by way of apoptosis. It has been shown that mice lacking p53, an important factor for apoptotic signaling, accumulate significantly more skin tumors than wild-type mice after chronic exposure to broadband UVB (Li *et al.*, 1998). Apoptosis serves an essential role in response to sunburn, although it is generally shown to be an anti-inflammatory or immunosuppressive event (Voll *et al.*, 1997; Freire-de-Lima *et al.*, 2006).

Although UVB-induced apoptosis has been extensively described as occurring in the skin after UVB damage, much less is known about the extent of necrosis that occurs following UVB damage to keratinocytes *in vivo*. *In vitro* studies have shown that exposing keratinocytes to narrowband UVB radiation produces fractions of immunostimulatory, necrotic cells (Annexin V[−], PI⁺) in addition to non-immunostimulatory apoptotic cells (Annexin V⁺, PI[−]; Lai *et al.*, 2009). Although *in vivo* studies have not explicitly shown necrosis occurring in the epidermis, it has also been described that apoptotic cells can progress to secondary necrosis if they are not properly cleared by phagocytic cells (Kono and Rock, 2008; Berghe *et al.*, 2010; Tabas, 2010). Many mediators of both apoptosis and necrosis, including TNF, are known to be stimulated following UVB exposure (Lai *et al.*, 2009; Bernard *et al.*, 2012; Galluzzi *et al.*, 2012). In this context, it could be speculated that sunburn leads to necrosis in the skin. Interestingly, it has recently been shown that HMGB1, a danger signal released from necrotic cells (Scaffidi *et al.*, 2002), is released from both cultured keratinocytes as well as keratinocytes present in murine skin following UVB exposure (Johnson *et al.*, 2013). Although this damage-associated molecular pattern has also been shown to be actively secreted from monocytes and macrophages (Wang *et al.*, 1999; Semino *et al.*, 2005), its immunostimulatory potential has been well described following necrosis (Scaffidi *et al.*, 2002; Kono and Rock 2008; Krysko *et al.*, 2008). HMGB1 has also been

shown to be released during pyroptosis, a specialized form of pro-inflammatory cell death (Lu *et al.*, 2012). Whether HMGB1 release from keratinocytes exposed to UVB is an active or passive process remains to be determined.

As necrosis has classically been described as an unregulated form of cell death in which membrane integrity is lost, it has only been observed *in vitro* in keratinocytes in which membrane permeability could be measured or morphology of a rupturing cell membrane could be observed. This has made observing necrosis *in vivo* difficult. In more recent years, however, necrotic cell death has been shown to be dependent on the activation of RIPK1 and/or RIPK3 and has earned the name necroptosis when dependence on these kinases is demonstrated (Cho *et al.*, 2009; Kaczmarek *et al.*, 2013). In addition, another specialized type of pro-inflammatory cell death known as pyroptosis, which is dependent on caspase-1 activation, may also occur after UVB damage to keratinocytes. It has been demonstrated that UVB radiation can stimulate inflammasome-dependent IL-1 β activation and secretion in keratinocytes (Feldmeyer *et al.*, 2007). This process is dependent on caspase-1 and can be induced by either inflammasome or pyroptosome formation (Fernandes-Alnemri *et al.*, 2007), which can lead to pyroptosis (Galluzzi *et al.*, 2012; Lian *et al.*, 2012). To what extent levels of apoptosis and necrosis or other forms of cell death are mediated after UVB exposure or whether one pathway is more prevalent at different UV doses is yet to be determined. It has been demonstrated, however, that increasing UVC and broadband UVB exposure causes dose-dependent increases in apoptosis (Rehmtulla and Hamilton, 1997; Mammone *et al.*, 2000). It has also been shown that higher doses of broadband UVB can induce necrosis in keratinocytes (Mammone *et al.*, 2000; Caricchio *et al.*, 2003). As not all cells in the epidermis visibly undergo apoptosis, although theoretically they receive the same amount of energy from UV radiation, it is possible that non-apoptotic forms of cell death occur in the epidermis after UV damage.

Once membrane integrity is lost during necrosis, cellular components from these damaged cells spill into extracellular spaces (Berghe *et al.*, 2010). These intracellular components are ‘foreign’ to an extracellular environment, and thus are treated as such by the immune system. A number of cellular products have been observed to stimulate PRRs, especially during necrosis, including HMGB1 (Scaffidi *et al.*, 2002), S100 proteins (Ryckman *et al.*, 2003), heat shock proteins (Basu *et al.*, 2000), ATP (Bours *et al.*, 2006), uric acid (Shi *et al.*, 2003), hyaluronan (Taylor *et al.*, 2004; Scheibner *et al.*, 2006), chromatin (Ishii *et al.*, 2001), and RNA (Karikó *et al.*, 2004; Cavassani *et al.*, 2008; Lai *et al.*, 2009; Bernard *et al.*, 2012). These molecules, some of which are normally confined to the interior of a cell, gain the ability to activate certain PRRs including Toll-like receptors. It has been demonstrated that the PRRs TLR2, TLR3, TLR4, and TLR9 can recognize certain damage-associated molecular patterns and induce an immune response.

TLR3 SENSES CELLULAR DAMAGE FOLLOWING SUNBURN

TLR3 is a PRR that binds double-stranded RNA (dsRNA; Liu *et al.*, 2008). Although it has traditionally been accepted that

dsRNA is a marker of viral infection or replication (Weber *et al.*, 2006), more recent evidence demonstrates that endogenous sources of RNA can also activate TLR3. In 2004, Kariko *et al.* showed that TLR3 on dendritic cells could be activated by RNA associated with necrotic cells and also by *in vitro* transcribed mRNA (Karikó *et al.*, 2004). In this study, when necrotic cells or mRNA were treated with benzonase, a nuclease that degrades all DNA and RNA, the necrotic cells no longer showed the ability to activate inflammatory pathways. In 2008, Cavassani *et al.* (2008) confirmed these findings *in vivo* by demonstrating that less inflammation was present in sterile gut injury models in *Tlr3*^{-/-} mice. They also showed that macrophages treated with necrotic cells needed functional Tlr3 to produce chemokines. In addition, they demonstrated that macrophages treated with apoptotic cells produced significantly less cytokines than when treated with necrotic cells (Cavassani *et al.*, 2008). This study demonstrates that if RNA is confined to intracellular compartments, as in apoptotic cells, it is unable to activate Tlr3. Only when RNA leaves the confines of the cell membrane does it become immunostimulatory.

In 2009, Lai *et al.* demonstrated that in a sterile wound model in the skin, TNF and IL-6 production was diminished in *Tlr3*^{-/-} mice (Lai *et al.*, 2009). This publication also demonstrated that narrowband UVB-damaged keratinocytes when added to keratinocyte cultures could stimulate the inflammatory cytokines TNF and IL-6 through the activation of TLR3. When the UVB-damaged normal human epidermal keratinocytes were treated with RNase, they no longer induced inflammatory cytokines (Lai *et al.*, 2009). In 2011, Lin *et al.* demonstrated that Tlr3 activation was also important for wound healing in the skin. They showed that *Tlr3*^{-/-} mice displayed a delay in wound healing, showing deficiencies of infiltrating neutrophils and macrophages (Lin *et al.*, 2011). They also were able to demonstrate that by applying the TLR3 ligand Poly (I:C), a dsRNA mimetic, to the wounds of mice as well as humans, wound healing was accelerated (Lin *et al.*, 2012). These studies suggest that RNA released from damaged keratinocytes induces TLR3-

dependent inflammation that promotes tissue repair. It has also been observed that activation of TLR3 in keratinocytes leads to increases in epidermal lipid transport and lipid metabolism gene expression, lipid accumulation, and increased lamellar bodies (Borkowski *et al.*, 2013). Because epidermal lipids are essential for proper barrier function and must be regenerated following injury to the epidermis, it can be speculated that activation of TLR3 in the skin helps to restore proper barrier function following UV injury.

Although the phenomenon that necrotic cells could stimulate TLR3 had been published multiple times in multiple cell types, it was not until 2012 that an endogenous ligand for TLR3 was discovered. Bernard *et al.* demonstrated that narrowband UVB damage to keratinocytes released U1 RNA, a noncoding small nuclear RNA and component of the spliceosome, that could act as a DAMP and activate TLR3 to induce the inflammatory cytokines TNF and IL-6. Bernard *et al.* used RNA sequencing to determine that U1 RNA was significantly increased in keratinocytes 24 hours after narrowband UVB exposure. It was also discovered that, in addition to U1 RNA, numerous additional noncoding RNAs were increased in keratinocytes after narrowband UVB exposure (Bernard *et al.*, 2012). Although these studies focused on the effects of U1 RNA, it is possible that any of these noncoding RNAs could act as damage-associated molecular patterns.

dsRNA is the required ligand for TLR3 activation (Liu *et al.*, 2008). Therefore, for an endogenous single-stranded RNA to be recognized by TLR3, it must form double-stranded regions. U1 RNA has a secondary structure comprised of four double-stranded stem-loop regions. These double-stranded regions serve to activate TLR3. The addition of U1 RNA or only a single stem-loop region of U1 RNA to keratinocytes was sufficient to induce TNF. Also, U1 RNA injected into ears of mice only induced inflammation when functional Tlr3 was present (Bernard *et al.*, 2012). It is believed that UVB damage to keratinocytes causes necrosis and that RNA released from necrotic keratinocytes can signal in a paracrine manner to induce inflammation in the skin through TLR3 on neighboring cells (Figure 1).

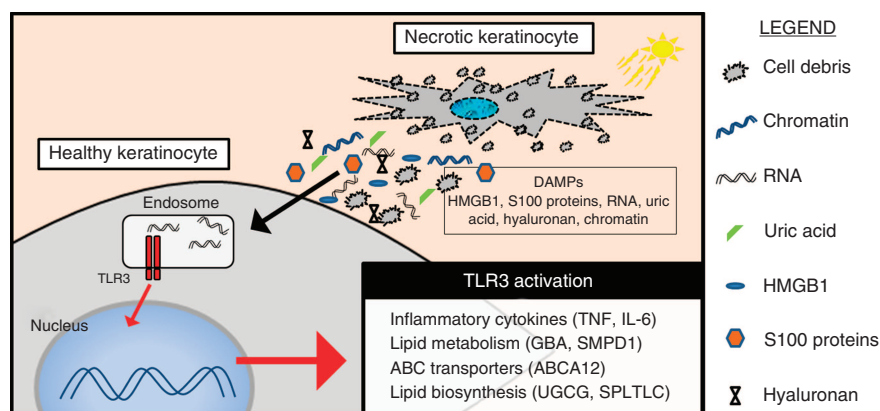


Figure 1. Double-stranded RNA from UV-damaged keratinocytes activates TLR3. Excessive UV exposure causes necrosis in a population of keratinocytes in the epidermis. Loss of membrane integrity in these necrotic keratinocytes causes cellular contents to be spilled into extracellular space. Damage-associated molecular patterns released by necrotic keratinocytes are then taken up by neighboring, healthy keratinocytes. Double-stranded RNA from necrotic keratinocytes is trafficked to the endosome where it activates TLR3. Downstream signaling leads to inflammation and increases in lipid biosynthesis, metabolism, and transport.

TLR3 is not the only TLR that has been implicated in the host response to UVB damage. Although it is believed that dsRNA released from necrotic keratinocytes has a role in this response, it is possible that other cell types in the skin can also undergo necrosis and contribute to this phenotype. It has been demonstrated that TLR3 and TLR4 activation can cause necrosis in macrophages that are treated with pan-caspase inhibitors (He *et al.*, 2011). TLR4, which can be activated by numerous damage-associated molecular patterns including hyaluronan (Taylor *et al.*, 2004; Scheibner *et al.*, 2006; Muto *et al.*, 2014), HMGB1, uric acid, heat shock proteins, defensins, and the bacterial ligand lipopolysaccharide (Kono and Rock, 2008), has also been shown to have a role in UVB-induced immune suppression, as *Tlr4*^{-/-} mice fail to exhibit UVB-induced immunosuppression in a contact hypersensitivity model (Lewis *et al.*, 2011). The same phenotype is seen in *Tlr3*^{-/-} mice, which also fail to show immunosuppression to DNFB contact hypersensitivity when first exposed to narrowband UVB radiation (Bernard *et al.*, 2012). There is also evidence that TLR4 may have a detrimental role in the response to UVB as *Tlr4*^{-/-} mice show increased nucleotide excision repair in skin (Ahmad *et al.*, 2014) after UVB exposure and higher rates of survival in *Tlr4*^{-/-} macrophages exposed to UVB (Harberts *et al.*, 2013). The role of TLR4 in UVB-induced cutaneous carcinogenesis remains to be tested and it would be interesting to see whether *Tlr4*^{-/-} mice have a different incidence of tumor formation. It has been shown, however, that chemically induced cutaneous carcinogenesis is in part dependent on Tlr4 (Mittal *et al.*, 2010) and downstream MyD88 signaling (Cataisson *et al.*, 2012). No studies to date have investigated the role of TLR3 on UVB-induced skin cancer.

REGULATION OF INFLAMMATION IN THE SKIN AFTER UVB INJURY

If damage to the epidermis is constantly occurring either by UVB exposure or mechanical injury, one might pose the question as to why the epidermis is not in a constant inflammatory state. It has been shown that TLR2 activation by commensal microbial ligands can dampen TLR3-induced inflammation (Lai *et al.*, 2009). In these studies, Lai *et al.* demonstrated that lipoteichoic acid from *Staphylococcus epidermidis*, a major component of the human skin microbiome, can dampen TLR3 signaling in keratinocytes and mouse skin. In these studies, TLR2 activation with lipoteichoic acid increased levels of the signaling molecule TRAF1, which is proteolytically processed by caspase-8 to its active form (N-TRAF1). N-TRAF1 can then bind TRIF to negatively regulate the TLR3-TRIF signaling pathways. The suppressive effects of lipoteichoic acid on TLR3-dependent inflammation were not seen in *Tlr2*^{-/-} and *Traf1*^{-/-} mice, showing that TLR2 is essential for mediating TLR3-induced inflammation in the skin. Interestingly, *Traf1* levels in skin were significantly lower in germ-free mice than in conventionally raised mice (Lai *et al.*, 2009). In this regard, commensal microbes such as *S. epidermidis* have the potential to suppress excessive inflammation in the skin following UVB damage or mechanical injury and the

presence of specific microbes may keep excessive inflammation in check.

Inflammation in the skin serves an important purpose. Inflammation following infection or injury helps to sterilize the wound and promotes wound healing by attracting leukocytes that attack microbes or clear apoptotic and necrotic debris (Martin and Leibovich, 2005). On the other hand, it has been demonstrated that certain innate immune pathways, those dependent on MyD88 signaling can promote chemically induced skin carcinogenesis (Cataisson *et al.*, 2012). It has also been demonstrated that *Il-10*^{-/-} mice are resistant to broadband UVB-induced tumorigenesis (Loser *et al.*, 2007). IL-10, which has been shown to be an important immune suppressor, induced following UVB irradiation, has been shown to contribute to UVB-induced skin cancer. Although inflammation may help clear infections and resolve wounds in the short term, its role in skin carcinogenesis must be further elucidated as mice deficient in both pro- and anti-inflammatory machinery develop fewer tumors in different models of carcinogenesis. Study of inflammatory pathways should continue to be an important field of research as a balance of short-term and long-term health effects of UVB is further elucidated.

CONCLUSIONS

Because skin serves as the interface of our bodies to the outside world, it constantly comes in contact with microbes and is at highest risk for injury. It is not unfathomable that common receptors exist to deal with distinctly different threats to our well-being. As TLR3 has been demonstrated to be important for inflammation in the skin following injury, the downstream pathways of TLR3 signaling must be further elucidated in order to develop therapeutics that take advantage of the beneficial effects without the adverse effects of excessive inflammation. As Toll-like receptors continue to be investigated, they are found to be implicated in a growing number of cellular processes. Recently, TLR3 has been linked to itch (Gruber and Holtz, 2013) as well as cardiac dysfunction following polymicrobial sepsis (Gao *et al.*, 2012) and will likely continue to be implicated in more disorders in the future. Identifying additional mechanisms of TLR3 activation and further elucidating downstream signaling will give us a better understanding of this pathway and lead to better therapeutics. Toll-like receptors and elucidating their expanding roles in various tissues should remain a focal point of future research.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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